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Phytochemical Determination and Antibacterial Activity of *Punica granatum* Peel Extracts against Plant Pathogenic Bacteria

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Abstract

Plant pathogenic bacteria are recognized to be harmful microbes able to decrease the quantity and quality of crop production in the world. *Punica granatum* peel was screened for its potential use as biological control agent for plant pathogenic bacteria. *P. granatum* peel was successfully extract using n-hexane, methanol and ethyl acetate by maceration. The highest yield obtained by ethyl acetate showed that ethyl acetate extracted more compounds that readily soluble to methanol and n-hexane. For *in-vitro* antibacterial activity, three different species of plant pathogenic bacteria were used namely *Erwinia carotovorum* subsp. *Carotovorum*, *Ralstonia solanacearum*, and *Xanthomonas gardneri*. For all crude extracts, four different concentrations 25, 50, 100 and 200 mg/ml were used in cup-plate agar diffusion method. Streptomycin sulfate at concentration 30 µg/ml was used as positive control while each respective solvent used for peel extraction was used as negative control. The results obtained from *in vitro* studies showed only ethyl acetate extract possessed antibacterial activity tested on the plant pathogenic bacteria. Methanol and n-hexane did not show any antibacterial activity against plant pathogenic bacteria selected where no inhibition zones were recorded. *R. solanacearum recorded* the highest diameter of inhibition zones for all range of concentrations introduced followed by *E. carotovorum* subsp. *Carotovorum* and *X. gardneri*. For the minimum inhbitory concentration (MIC) and minimum bactericidal concentration (MBC), only the ethyl acetate extract was subjected to the assay as only ethyl acetate extract exhibited antibacterial activity. The minimum concentration of ethyl acetate extract that was able to inhibit plant pathogenic bacteria was recorded at a concentration of 3.12 mg/ml which inhibited *R. solancearum* and *E. carotovorum subsp. Carotovorum*, followed by *X. gardneri* at concentration 6.25 mg/ml. For the minimum bactericidal concentration (MBC), the results showed that at

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the concentration of 12.5 mg/ml, the extract was still capable of killing the pathogenic bacteria, *R. solanacearum*, and *P. caratovora* sub.sp. *caratovora* while for the bacteria *X. gardneri*, the concentration that was able to kill the bacteria was 25 mg/ml. The qualitative estimation of phytochemical constituents within *P. granatum* L. ethyl acetate peel extracts had revealed the presence of tannins, flavonoids, phenols alkaloid, Saponins, and terpenoids. This study has demonstrated that Ethyl Acetate peel extracts of *P. granatum* has significant antibacterial activity against pathogenic plant bacterial, and it could be of high agricultural value.

Keywords

***Punica granatum*, Plant Extraction, Pathogenic Bacteria, Phytochemical Screening**

1. Introduction

For several years now, plants and plant materials have been used as a source of medicinal agent and numerous natural products acquired from medicinal plants either as a crude extract or as purified products have been employed in disease control. Medicinal plant parts have been extensively used to extract raw drugs owing to possession of various medicinal properties. They constitute credible sources for a huge number of modern drugs, several of which are usually based on their traditional folk medicine. The World Health Organization (WHO) has stated that medicinal plants are the best source for obtaining a variety of therapeutic agents, and several medicinal plants have been employed as a source of medicine in daily life for treatment of various types of ailments globally [1]. Clinical microbiologists have greatly used medicinal plants for the screening of new therapeutic agents [2]. A great range of biotic molecules referred to as secondary metabolites are produced by plants [3], thereby making them a rich source for diverse forms of medicine. Additionally, the primary advantage of using these naturally derived products include safety for human consumption, possess no harmful effects on the environment, and low cost is incurred in treating microbial infections when they are used [4]. In this study, *Punica granatum* were chosen as the antibacterial agent against plant pathogenic bacteria because these plants contain many biological activity potentials of being anti-diarrheal, antimicrobial, antimalarial, hepatoprotective effects, wound healing and many more. *Punica granatum* L. back to the family *Punicaceae* usually known as Pomegranate. It has just two species *P. protopunica Balf* and *P. granatum* Linn. *Punica granatum* is a plant or small tree with some upright, prickly stems, the leaves are roughly 2 × 1 inches, elliptic, flowers white or red, double-flowered races being also known. *P. granatum* is reported to be used for many disease conditions in traditional medicine. Some of its reported uses include enhancement of semen formation, gastrointestinal problems, memory activation, boosting of hemoglobin. Various parts of the plant are active in the management of many diseases such as dyspepsia, leprosy, bronchitis and hypertension. The plant has also been used as an antispasmodic and anthelmintic. In Hausa land, the flowers are used as vermifuge. The fruit and bark have also been used in tanning in ancient times. The plant is reported to contain over 28% of Gallotannic acid and the alkaloid pelletierine, methypelletierine, isopelletierine, gallic acid, psuedopelletierine, calcium oxalate, tannic acid, sugar [5]. In addition, the main advantage of using these naturally derived products are that they are safe to human health, do not leave any harmful effects on the environment and incur little cost in counteracting microbial infections [4]. *R. Solanacearum*, *X. gardneri*, and *E. caratovora sub.sp. caratovora* are believed to be the most important plant pathogens that are becoming destructive when affecting particular crops that strengthen the agriculturist trust that these plant pathogenic bacteria cannot be left to spread widely amongst the valuable crops that will lead to severe crop and economic losses. All of these pathogenic bacteria have treated as early as possible before the whole crops are damaged. These pathogens started to be resilient towards the antibiotics, and chemical pesticide used, so the use of chemical pesticides and antibiotics in controlling these destructive plant pathogens seem to be less effective. To face this problem, the use of *P. granatum* L. peel extracts could be another successful method in controlling severe plant diseases. For that reason, the mean objective of this study was to determine the antibacterial activity of *P. granatum* L. peel extracts against plant pathogenic bacteria.

2. Materials and Methods

2.1. Bacteria Isolate

All plant pathogenic bacteria used in this study were obtained from University Protection, the Microbiology Laboratory. They are *X. gardneri*, *R. Solanacearum*, *E. caratovora sub.sp. caratovora.* The cultures were maintained on nutrient agar (NA) for continued viability.

2.2. Plant Materials

Fresh fruit of pomegranate (*P. granatum* L.) was collected from the local market. The fruit was washed with running tap water to remove dirt and insects from the peel surfaces and then separate the seeds from the peel and then dried in an oven at 50˚C for three days. Then crushed into the fine peel powder using a cross beater mill (SK100, Retsch) machine with sieving size 0.50 mm (SK100, Retsch). Three hundred gm. of *P. granatum* peel powder placed into three separate (2 L) volume conical flask labeled with n-hexane, methanol, and ethyl acetate. Tow liters each of n-hexane, methanol, and ethyl acetate were poured into different labeled flask. The flask was shaken gently to mix well the organic solvents with the peel powder and was left under fume chamber for two days with occasional shaking. The extracts were filtered through Whatman No.1 filter paper with an aid of aspirator (A-3S, Eyela) into three different clean flasks. The filtrates were then lyophilized using rotavapor. The dry extracts were then collected into a small beaker and labeled based on the organic solvents used. The extracts then were kept in chillers (Chill-300, Protech) at 4˚C for further use slight modification from [6] and [7].

2.3. Antibacterial Activity of *P. granatum* Peels Extracts

The effects of *P. granatum* peel extracts on bacterial growth were measured by *in vitro* agar well diffusion assay according to the technique described by [8] with slight modification. 150 µl of standardized bacterial suspension with O.D. = 0.1 was spread over 20 mm thick Mueller Hinton Agar (MHA) with an L-shaped glass rod and left for 5 minutes to dry. Six wells with 0.6 mm diameter were. Each well was filled with 50 µl of extracts different concentration of 200, 100, 50 and 25 mg/ml. The other two empty wells were filled with 50 µl of Streptomycin sulfate (30 µg/ml) as a positive control and 50 µl of absolute methanol as a negative control. All plates were then incubated at 30˚C for 24 h and were done in three replicates. Measured with a ruler in millimeter (mm) the zones around the wells were will record as inhibition zone for extracts.

2.4. Statistical Analysis

Data analysis, all variables were subjected to normality test, and the results showed that all variables were normally distributed. To compare different concentration (treatment) data were analyzed by analysis of variance (ANOVA) based on CRD (completely randomized design) using SAS version 9.1. The mean comparison was done by Duncan Multiple Range Test (DMRT). The significant differences were considered significant at $P < 0.05$ [9]. The results of IC_{50} and IC_{90} values were calculated using probit analysis using Polo Plus Ver 2.

2.5. Minimal Inhibitory Concentration (MIC)

Minimal inhibitory concentration (MIC) determination, the lowest concentration (25 mg/ml) of the antibacterial activity that was still able to inhibit the bacteria was taken as the starting concentration for making dilution. Eleven test tubes labeled (T1-T11) were each filled with 1 ml Mueller-Hinton Broth (MHB) for each bacterial isolate. Two-fold serial dilutions (1:1) were made by filling in 1 ml of the extracts to the first test tube and vortexed to mix the solution well. After that take 1 ml of the solution from T1 was withdrawn and diluted into (T2)a second test tube and repeated the procedure until test tubes 10 (T10). 100 µl of the standardized bacterial suspension was then inserted into each test tube except for T10 that served as positive control (MHB + plant extracts only). The last test tube (T11) served as t h e negative control (MHB + bacteria suspension). Finally, 50 µl of 2,3,5-Triphenyltetrazolium chloride (TTC) was added to each test tube. All the test tubes were then incubated for 24 h at 30˚C. after that any test tube that did not change into red color was logged as the MIC.

2.6. Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration value was determined by a subculture the test tubes from MIC essay

that did not change into a red color, onto the sterile MHA plate. Then incubated overnight at 28˚C. The least concentration that presented no detectable growth on the agar plates was considered as the MBC value [10].

2.7. Protocol for Phytochemical Screening

The phytochemical screening of *P. granatum* L. ethyl acetate peel extracts was done to find the presence of active chemical constituents such as Flavonoids, Alkaloids, Saponins, Tannins, Phenolic, and terpenoids. The phytochemical screening was done based on previous procedures published somewhere else [11]-[18].

3. Results

3.1. *In-vitro* Antibacterial Activity of *Punica granatum* Peel Extracts

As the result shown, there was no antibacterial activity of *Punica granatum* methanol and n-hexane crude extracts detected against all species of plant pathogenic bacteria. The antibacterial activity of *P. granatum* L. ethyl acetate peel extracts was successfully evaluated *in-vitro* against three plant pathogenic bacteria. They are *X. gardneri*, *R. Solanacearum*, *E. caratovora sub.sp. caratovora*. These bacteria are known to cause a harmful effect on valued food crops. Table 1 and Figure 1 show inhibition zone diameter ranging from (8.50 ± 0.58 mm) to (22.75 ± 0.48 mm). As the positive control Streptomycin sulfate (30 µg/ml) showed inhibition towards three bacterial isolates ranging from (24.75 ± 0.48 mm) to (26.75 ± 0.48 mm). There was no inhibition recorded for all plant pathogenic bacteria when tested with absolute ethyl acetate as the negative control.

At the lowest concentration (25 mg/ml) of extracts used, the highest mean of inhibition zone was recorded for *R. Solanacearum* (13.0 ± 0.58^{a} mm), followed by then *E. caratovora sub.sp. caratovora* (9.5 ± 0.29^{e}) and *X. gardneri* (each 8.5 ± 0.58 mm). At a concentration of 50 mg/ml, the lowest mean diameter inhibition zones were recorded for *X. gardneri* (12.5 ± 0.29 mm) followed by *E. caratovora sub.sp. caratovora* (13.50 ± 0.29 mm)

Table 1. Means (n = 4) of inhibition zone diameter (mm) of *P. granatum* L. ethyl acetate peel extract against plant pathogenic bacteria. Within rows, values with different letters differ significantly.

Concentration	*R. solanacearum*	*X. gardneri*	*E. carotovora*
25	13.0 ± 0.58^{c}	8.5 ± 0.29^{f}	9.5 ± 0.29^{e}
50	15.75 ± 0.48^{d}	12.5 ± 0.29^{d}	13.50 ± 0.29^{d}
100	19.25 ± 0.48^{c}	15.5 ± 0.29^{c}	18.50 ± 0.29^{c}
200	22.75 ± 0.48^{b}	19.5 ± 0.29^{b}	21.75 ± 0.25^{b}
Positive Control	26.75 ± 0.48^{a}	24.75 ± 0.48^{a}	25.0 ± 0.41^{a}
Negative Control	0^{f}	0^{f}	0^{f}

(a) (b) (c)

Figure 1. Inhibition zone diameter of *Punica granatum* L. ethyl acetate peel extracts against plant pathogenic bacteria. (a) *E. caratovora sub.sp. caratovora*; (b) *R. Solanacearum*; (c) *X. gardneri*.

while *R. Solanacearum* showed diameter inhibition zone of (15.75 ± 0.48). While a concentration of 100 mg/ml, the highest mean diameter inhibition zones was recorded for *R. Solanacearum* (19.25 ± 0.48), *E. caratovora sub.sp. caratovora* showed diameter inhibition zone of (18.50 ± 0.29 mm) while *X. gardneri* (15.5 ± 0.29 mm). At the highest extract concentration of 200 mg/ml, the highest mean diameter was recorded for *R. Solanacearum* (22.75 ± 0.48 mm), followed by *E. caratovora sub.sp. caratovora* (21.75 ± 0.25 mm) and *X. gardneri* showed diameter inhibition zone of (19.5 ± 0.29 mm).

3.2. Minimum Inhibitory Concentration (MIC)

The (MIC) all the tested bacterial isolates, the lowest concentration that still inhibited the plant pathogenic bacteria was at 3.125 mg/ml for *R. Solanacearum* followed by *E. caratovora sub.sp. caratovora* with the lowest concentration recorded at 3.125 mg/ml while *X. gardneri* with the lowest concentration recorded at 6.25 mg/ml. For MBC, The lowest concentration that showed no visible growth on the agar plates was considered the MBC value 25 mg/ml recorded for *X. gardneri* while concentration 12.5 mg/ml for *R. Solanacearum* and *E. caratovora sub.sp. caratovora* as shown in Table 2 and Figure 2.

The results of IC_{50} and IC_{90} values were calculated using probit analysis using Polo plus Ver 2 and results indicated that the chi square test for all these three bacteria has significant heterogeneity in the test population (Table 3). The highest IC_{50} was observed for *X. gardneri* followed by *E. carotovorum* and *R. solancearum*. This results indicated the *X. gardneri* had the highest IC_{90}.

Table 2. Minimal inhibitory concentration (MIC) and minimum bactericidal Concentration (MBC) of *P. granatum* L. ethyl acetate peel extracts against plant pathogenic bacteria by macro-broth dilution methods.

Bacteria species	MIC (mg/ml)	MBC (mg/ml)
Ralstonia Solanacearum	3.125	12.5
Erwinia caratovora sub.sp. caratovora	3.125	12.5
Xanthomonas gardneri	6.25	25

Table 3. Probit regression line parameter and inhibition concentration (IC).

Bacteria	Regression equation	Chi-square (df)	IC_{50} with fiducial limits	IC_{90} with fiducial limits
R. solancearum	Y = 1.086 - 1.644 X	0.156 (2)	32.55 (5.8 - 55.2)	492.25 (197.9 - 39033.0)
X. gardneri	Y = 1.207 - 2.181 X	0.050 (2)	63.99 (32.8 - 111.6)	737.11 (276.6 - 39355.0)
P. carotovorum	Y = 1.570 - 2.625 X	0.063 (1)	46.971 (25.8 - 68.4)	307.64 (167.7 - 1622.8)

Figure 2. Minimum inhibitory concentration (MIC) of *Punica granatum* L. ethyl acetate peel extract against *X. gardneri* (A), *R. solancearum* (B), *E. carotovorum* (C). (T1 = 25 mg/ml, T2 = 12.5 mg/ml, T3 = 6.25 mg/ml, T4 = 3.125 mg/ml, T5 = 1.563 mg/ml, T6 = 0.781 mg/ml, T7 = 0.391 mg/ml, T8 = 0.195 mg/ml, T9 = 0.098 mg/ml, T10 = positive control (MHB + plant extract), and T11 = Negative control (MHB + bacteria suspension). The results obtained from macro broth dilution technique using test tubes were transferred into microliter plate for the photography purpose.

3.3. Phytochemical Screening of *P. granatum* L. Ethyl Acetate Peel Extracts

The phytochemical screening of *P. granatum* L. ethyl acetate peel extracts the presence some major compound known as secondary metabolites determine, the results obtained were shown in Table 4. It showed the positive results for the presence of alkaloid, saponin, phenol, tannin, flavonoid, terpenoid. As determined by the color change.

4. Discussion

P. granatum L. against plant pathogenic bacteria were effective against all plant pathogenic bacteria tested. This can probably replace the use of synthetic antibiotics as they can become resistance to the antibiotics when used. Most of the previous research *P. granatum* L. is reported to be used for many disease conditions in folktale medicine. Previous studies showed that some researchers like [19]-[21] stated that Punica granatum peel extracts in different concentrations were effective against streptococci strains (streptococci *mutans*; streptococci *aureus*; streptococci *salivarius*; streptococci *sanguinis* and streptococci *epidermidis*). It was confirmed that this antibacterial activity may be connected to the presence of polyphenolics and hydrolysable tannins in the pomegranate extract specifically gallagic acid and punicalagin [22] [23]. It means that the antimicrobial influence of tannins was linked to its toxicity and structure of molecular. Tannins may be a performance on the cell wall and across the cell membrane because they can precipitate proteins [23] [24]. They may also suppress many enzymes such as glycosyltransferases [21] and [20] demonstrated that gallic acid (a tannic acid) has the highest antibacterial effect against tested sensitive strains even at low concentrations. Hence, the antibacterial activity of Punica granatum may be related to polyphenol structures because polyphenols may affect the bacterial cell wall, inhibit enzymes by oxidized agents, interact with proteins and disturb coaggregation of microorganisms [20] [21]. This study proved that *P. granatum* Ethyl Acetate peel extracts can be industrialized as next antibacterial agents to control plant pathogenic bacteria as it was able to inhibit the growth of plant pathogenic bacteria. All bacteria sp. isolates gave good inhibition zones when tested with all range of concentration. Another study has reported that pomegranate extract inhibited *Pseudomonas aeroginosa* growth and had an interactive effect against bacteria resistant to the known antibiotics. Synergistic effects of methanolic pomegranate extract and chloramphenicol, gentamicin, ampicillin, tetracycline and oxacillin against *Staphylococcus aureus* is also reported by [25]. [26] reported that the antibacterial activity of peel extracts of *pomegranate* against both Gram positive and negative bacteria strains and mention MIC values ranging from 0.25 to 4.0 mg/ml against the tested bacteria. Also, he reported a two-fold MIC value against a Gram-positive bacterium (*S. aureus*) than against a Gram-negative bacterium (*E. coli*). The phytochemical screening of Punica granatum L ethyl acetate peel extracts, the results obtained were shown the positive results for the presence of flavonoid, alkaloid, saponin, terpenoid, phenol, tannin. Study for [27] reported that the *pomegranate* peel indicated the presence of Alkaloid, Tannins, Flavonoids, Saponins, Glycosides, Sterols, Resins, Volatile oils, Carbohydrates, Balsams, Terpenes, and Free- Reducing sugar. The presence of these metabolites suggests great possibilities for the plant as a source of useful phytomedicines. For instance, some alkaloids are known to be used as antimalarial agents [28]. Tannins too could show that it is a severe, help in wound healing and anti-parasitic. The presence of resins and flavonoids might be responsible its use as anti-inflammatory properties [29]. The presence of terpenes proposes it possible use as an antiviral and antitumor [30].

Table 4. Phytochemical screening of *Punica granatum* L. ethyl acetate peel.

Chemical Constituents	Positive Results	Ethyl Acetate extracts
Saponin	Small bubbles (foam)	+
Alkaloid	Creamish precipitate	+
Phenol	Dark blue	+
Tannin	Blue-green color	+
Flavonoid	Dark yellow	+
Terpenoid	Reddish brown coloration	+

5. Conclusion

P. granatum Ethyl Acetate peel extracts gave significant antibacterial activity against *X. gardneri*, *R. Solanacearum*, *E. caratovora sub.sp. caratovora*, which indicate that these extracts have the potential being the next natural biological control agent for plant pathogenic bacteria. The main compounds found in this study can be used to produce new and useful natural chemical product as an alternative to the use of synthetic antibiotics and chemicals for the control of plant pathogenic bacteria.

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Original Article

Antibacterial and Antifungal Activities of Punica Granatum Peel Extracts Against Oral Pathogens

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Abstract:

Objective: *Punica granatum* has been used for many years in folk medicine due to several purposes. The aim of the present study was to evaluate the effect of methanolic extract of *Punica granatum* peel (MEPGP) against *Streptococcus mutans*, *Staphylococcus aureus*, *Streptococcus salivarius*, *Streptococcus sanguinis*, *Staphylococcus epidermidis*, *Actynomyces viscosus*, *Lactobacillus acidophilus* and *Candida albicans*.

Materials and Methods: In this *in vitro* study, the mentioned oral organisms were cultured in blood agar and mueller-hinton media and then paper disks containing MEPGP at concentrations of 4 mg/ml, 8 mg/ml and 12 mg/ml were inserted on medias. The antimicrobial activity was evaluated by agar disk diffusion method. The effects of three different concentrations of MEPGP against microorganisms were compared using one-way ANOVA and Tukey tests.

Results: All concentrations of MEPGP had antibacterial activity against *S. aureus* and *S. epidermidis*. Only at concentration of 8 mg/ml and 12 mg/ml MEPGP was effective against *L. acidophilus*, *S. mutans* and *S. salivarius*. Furthermore, no concentrations of MEPGP inhibited *A. viscosus* and *C. albicans*.

Conclusion: This study suggests that MEPGP might be used as an antibacterial agent in controlling oral infections.

Key Words: Punicaceae; Anti-Bacterial Agents; Antifungal Agents; *Staphylococcus aureus*; *Staphylococcus epidermidis*; *Lactobacillus acidophilus*; *Streptococcus mutans*; Streptococcus; *Candida albicans*; *Actinomyces viscosus*

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INTRODUCTION

In the recent decades, the interest in evaluating therapeutic effects of plants has increased dramatically [1,2] as 80% of the world's people rely on complementary and alternative medicine for their health care needs [3,4]. Phytoplants have been shown to be good alternatives to synthetic chemical antimicrobial agents and antibiotics because of the serious side effects, antimicrobial resistance and the emergence of previously uncommon infections that have been reported to be on the increase due to inappropriate or widespread overuse of antimicrobials [5,6]. On the other hand, clinicians should remind the potential risk of urticaria, alteration in taste, increase of calculus

formation, staining of teeth and mucous membranes and more rarely, oral mucosa desquamation and parotid swelling before prescribing chlorhexidine mouth wash as an antimicrobial agent [7,8]. However, from 250,000-500,000 natures found on earth, only one percent of them have been assessed for their pharmaceutical potential [9]. There are manuscripts proving the effects of Iranian native herbal extracts [10-13]. *Punica granatum* (pomegranate) is native to the region from northern India to Iran. But it is also widely cultivated now in parts of Southwest America, California, Mexico, Arizona and Africa [14,15]. Pharmacological effects of pomegranate represent a long history and have been mentioned in the Greek and Egyptian documents [16,17]. Recently, studies have shown that pomegranate has many potential effects including: bacteriocidal, antifungal, antiviral, immune modulation, vermifuge, stimulant, refrigerant, astringent, stomachic, styptic, laxative, diuretic and anthelmintic. Moreover, it serves to decrease the adverse effects of cardiovascular diseases, diabetes, diarrhea, dysentery, asthma, bronchitis, cough, bleeding disorders, fever, inflammation, acquired immune deficiency syndrome, dyspepsia, ulcers, bruises, sores, mouth lesions, skin lesions, malaria, prostate cancer, atherosclerosis, hypertension, periodontal diseases, hyper lipidemia, denture stomatitis, male infertility, vaginitis, erectile dysfunction, alzheimer, obesity and infant brain ischemia [4,9,14-17]. Furthermore, pomegranate is an amazing source of cyaniding, delphinidin (both are anthocyanidins), caffeic acid, chlorogenic acid (both are phenolic acids), gallic acid, ellagic acid (tannic acids), luteolin, quercetin (flavones), kaempferol (a flavonol), naringenin (a flavanone) as well as 17-alpha-estradiol, estrone, estriol, testosterone, beta-sistosterol, coumesterol, gamma-tocopherol, punicie acid, campesterol and stigmasterol in its juice, peels and seed oil that are chemopreventive and therapeutic potentials of this plant [14,18]. Review of the literature from 1999 to the present showed that scientific papers relating to the therapeutic effects of pomegranate are increasing compared to only 25 publications from 1950 to 1999 [14,15]. Since pomegranate has varied medical effects, it should be considered by researchers in different parts of medical sciences. Therefore, this study has been based on the antibacterial and antifungal properties of *Punica granatum* on different oral pathogens.

MATERIALS AND METHODS

Preparation of the Plant Extract

Firstly fresh pomegranates (500 gr) were obtained (in order to prepare fresh extraction) from a public market in the city of Hamadan, Iran. The peels of pomegranate were separated and oven dried at 33°C for 7 days. The dried peels were powdered in an electric grinder and stored in plastic bags for the next step. A 100 gm sample of powder was extracted using 200 ml methanol (99.9%) in an electric blender for 30 min. This suspension was filtered three times per day for 30 days. New methanol dissolvent was used each time. Then methanol was removed in a rotary evaporator to produce a dry powder. The final material was dissolved in methanol for obtaining concentrations of 4mg/ml, 8mg/ml and 12 mg/ml of dry plant powder [9,16,19]. Then specimens were sent to the institute of biological science for assessment of their antibacterial and antifungal effects.

Microorganisms

Type strains were obtained from American Type Culture Collection (ATCC) and Persian Type Culture Collection (PTCC) as follows:
Streptococcus mutans (PTCC 1683), *Streptococcus sanguinis* (PTCC 1449), *Streptococcus salivarius* (PTCC 1448), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (PTCC 1114), *Actinomyces viscosus* (PTCC 1202), *Lactobacillus acidophilus*

(PTCC 1643) and *Candida albicans* (PTCC 5027), which were all obtained from the microbiology laboratory of Hamadan University of Medical Sciences, Hamadan, Iran. Each of the bacterial specimens was incubated in liquid culture dilutions (Tryptone Soy Broth, Oxoid, British) and incubated at 37°C for 20 min to reach the logarithmic stage, then measured to a 0.5 Mc Farland dilution (standard concentrations) which delivered a final concentration of approximately 105 CFU per ml. Then the agar plates with methanolic extract of *Punica granatum* peel (MEPGP) were incubated over night at 37°C [20].

Antibacterial Tests

We tested MEPG at different concentrations using a standard diffusion technique [4,9,21-24]. The MEPGP samples were inserted in 6 mm sterile filter disks (Blank paper, Padtan Teb, Iran) and incubated for 20 min at 37°C and collected in sterile containers. The disks were then placed on the surface of blood agar and meuller-hinton agar (Merk, Germany), in which the microorganisms were cultured. Ciprofloxacin and nystatin disks served as the positive control and diluted methanol was used as the negative control. Finally, we measured the diameter of inhibition zones in millimeter after 24 hours. We placed four disks for each concentration in an 8 cm plate and calculated the mean of inhibition zones. One expert microbiologist conducted all the procedures. We analyzed the data using one-way ANOVA and Tukey test. P value less than 0.05 was considered statistically significant.

RESULTS

After evaluating the antibacterial and antifungal effects of three different concentrations of MEPGP, the positive control produced significantly large inhibition zones for all microorganisms and the negative control showed no markable inhibitory effect. It was found that all concentrations of MEPGP (4 mg/ml, 8 mg/ml and 12 mg/ml) inhibited *S. aureus* and *S. epidermidis*. In addition, the concentration of 12 mg/ml was the most effective extract against *S. aureus* compared with the others. Only two MEPGP concentrations of 8 mg/ml, 12 mg/ml were effective against *S. sanguinis*, *L. acidophilus*, *S. mutans* and *S. salivarius* and there was no significant difference between these concentrations (P=1, P=1, P=0.064). No concentrations of MEPGP inhibited *A. viscosus* and *C. albicans*. Detailed antibacterial and antifungal effects of MEPGP against eight certain oral pathogens are demonstrated in Table 1.

DISCUSSION

In the recent years, the use of plants with preventive and therapeutic effects contributes to health care needs [25]. There are three main reasons to be interested in the treating and healing power of plant extract. First, pharma-

Table 1. Antibacterial and antifungal properties of methanolic extract of punica granatum at three different concentrations

Microbial Strains	Antimicrobial Activity at*							P.Value	F
	Conc. of 4		Conc. of 8		Conc. of 12		Positive Control**		
	Mean	SD	Mean	SD	Mean	SD			
Staphylococcus aureus	7.5	0.57	11.5	0.56	12.5	0.58	30	0.000	155.66
Staphylococcus epidermis	11.5	0.57	13.5	0.59	13.5	0.58	29	0.000	20.00
Lactobacillus acidophilus	6.5	0.57	10.0	0.00	10.0	0	14	0.000	227.00
Actinomyces viscosus	6.0	0.00	6.5	0.57	6.5	0.57	25	0.168	2.00
Streptococcus mutans	6.0	0.00	9.5	0.57	9.5	0.57	24	0.000	98.00
Streptococcus sanguinis	6.5	0.57	10.0	0.00	11.5	0.58	25	0.000	172.00
Streptococcus salivarius	6.5	0.58	8.5	0.59	9.5	0.60	26	0.000	43.66
Candida albicans	6.0	0.00	6.5	0.57	6.5	0.57	40	0.168	2.00

* measured by the diameter of zone of inhibition in mm, Conc= Concentration, **Ciprofloxacin and nystatin are the positive control group

cological studies have demonstrated that many of plants are known to possess antimicrobial agents; second, people are becoming aware of the side effects associated with the over prescription of traditional antibiotics; third, time to time resistant microorganisms against antibiotics are increasing [9,22,25]. Among these plants, punica granate has an important role in folk medicine. Pomegranate is known as a rich source of pharmacological properties which have been evaluated due to antiparasitic, antibacterial, antifungal, antiproliferative, apoptotic and anti-cancer effects as well as protection against herpes virus, inhibition of LDL oxidation and decrease in atheromatous plaque formation and reduction of systolic blood pressure [17,18,22]. Results of the present study showed that MEPGP was effective against some common oral pathogens such as *S. epidermidis*, *S. aureus*, *L. acidophilus*, *S. mutans*, *S. sanguinis* and *S. salivarius*, but not effective against *A. viscosus and C. albicans*. Review of literature showed that some researchers such as Naz et al [22], Vasconcelos et al [26] and Singh et al [27] also reported that extracts of *Punica granatum* peel in different concentrations were effective against *S. epidermidis*, *S. aureus*, *S. mutans*, *S. sanguinis* and *S. salivarius*. It is demonstrated that this antibacterial activity may be related to the presence of hydrolysable tannins and polyphenolics in the pomegranate extract specifically punicalagin and gallagic acid [17,19]. It means that the antimicrobial effect of tannins is related to its toxicity and molecular structure. Tannins may act on the cell wall and across the cell membrane because they can precipitate proteins [19,26]. They may also suppress many enzymes such as gycosyltransferases [26]. Reddy et al [17] and Naz et al [22] demonstrated that gallic acid (a tannic acid) has the highest antibacterial effect against tested sensitive strains even at low concentrations. Hence, the antibacterial activity of *Punica granatum* may be related to polyphenol structures because polyphenols may affect the bacterial cell wall, inhibit enzymes by oxidized agents, interact with proteins and disturb co-aggregation of microorganisms [22,26]. In the present study, the extract of *Punica granatum* peel did not have an effect on *C. albicans* in all concentrations. This finding is in agreement with Singh's study [27], but is in disagreement with the report of Vasconcelos et al [26] and Duraipandiyan et al [4]. Vasconcelos et al showed that *Punica granatum* may be used as a topical antifungal drug against *C. albicans* in two reports [19,26]. The real mechanism of the antifungal effect of tannins (the major components of *Punica granatum* extract) against Candida albicans is not clear; however, it may be related to their toxicity, astringent, molecular structure or other ways [19,26]. Furthermore, in the present study *Punica granatum* extract had no effect on the growth of A. viscosus. It may be related to the fact that gram positive bacteria such as Actynomyces, Aspergillus flavus and Aspergillus parasiticus are more sensitive against antibacterial agents compared to gram negative bacteria because of the difference in their cell wall structures [27]. In the present study, we have used the disk diffusion method for the antimicrobial evaluation of MEPGP; however, the MIC method (Minimum Inhibitory Concentration) applied along with disk diffusion may be recommended in future studies.

CONCLUSION

Many herbs have preventive or therapeutic potentials. Therefore, further studies are required to find these effects in order to replace synthetic medications with natural remedies. According to the results of this study, the extract of *Punica granatum* might be used in the control of common oral pathogens responsible for caries, stomatitis and periodontal diseases. On the other hand, further photochemical studies are required to determine the type of compounds responsible for the antibacterial effect of pomegranate.

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ORIGINAL ARTICLE

Antibacterial Effect of *Granati fructus* Cortex Extract on *Streptococcus mutans In Vitro*

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ABSTRACT

The rind of pomegranate fruit (*Granati fructus* cortex) composed of antibacterial compounds such as alkaloid, flavonoid and tannin. **Objective:** To evaluate the bacterial effect of *Granati fructus* cortex extract against *Streptococcus mutans*. **Methods:** The study was laboratory experimental. The inhibition test was performed by agar diffusion method on MHA medium. **Results:** It showed the bacterial property of *Granati fructus* cortex on various concentration. The highest extract concentration of 30% extract has the largest of inhibition zones (15.4mm). The results showed a difference in the size of inhibition zones related to different extract concentrations. **Conclusion:** This study confirmed the antibacterial effect of *Granati fructus* cortex on the growth of *Streptococcus mutans*.

ABSTRAK

Efek antibakteri ekstrak kulit buah delima *(Granati fructus cortex)* pada *Streptococcus mutans in vitro*. *Granati fructus cortex* mengandung senyawa-senyawa antibakteri seperti alkaloid, flavonoid, dan tannin. **Tujuan:** Mengevaluasi efek antibakteri *Granati fructus cortex* dalam menghambat pertumbuhan *Streptococcus mutans*. **Metode:** Penelitian ini merupakan eksperimental laboratoris yang menguji daya hambat antibakteri menggunakan metode difusi agar dengan media MHA. **Hasil:** Ekstrak kulit buah delima dalam berbagai konsentrasi memiliki efek antibakteri, ekstrak kulit buah delima dengan konsentrasi 30% memiliki rata-rata zona hambat paling besar (15,4mm). Semakin tinggi konsentrasi ekstrak kulit buah delima maka semakin besar zona hambat yang terbentuk. Hasil uji ini juga menunjukkan adanya perbedaan rata-rata zona hambat dalam berbagai konsentrasi ekstrak kulit buah delima. **Simpulan:** *Granati fructus cortex* memiliki efek antibakteri terhadap pertumbuhan *Streptococcus mutans*.

Key words: antibacterial effect, *Granati fructus* cortex, *Streptococcus mutans*

PENDAHULUAN

Streptococcus mutans (*S.mutans*) adalah bakteri Gram positif yang dapat memetabolisme karbohidrat terutama sukrosa dan menciptakan suasana asam di rongga mulut.[1] *S.mutans* mempunyai dua sistem enzim pada dinding sel yang dapat membentuk dua polisakarida ekstraseluler dari sukrosa.[2] Sukrosa dihidrolisis menjadi fruktosa (levan) dan glukosa (dekstran). Fruktosa dihidrolisis oleh enzim fruktosiltransferase dan glukosa dihidrolisis oleh enzim glukosiltransferase.[1,3] Fermentasi sukrosa akan menghasilkan penurunan pH saliva menjadi 5,0 atau lebih rendah. Ketika pH plak turun di bawah 5,0, dapar saliva terganggu dan menyebabkan pelepasan ion kalsium dan fosfat dari kristal hidroksiapatit.[3]

Demineralisasi email dapat terjadi karena peningkatan konsentrasi asam laktat sehingga dapar saliva tidak cukup untuk mencegah larutnya email, selanjutnya proses karies dapat terjadi.[1,3] *S. mutans* merupakan bakteri penyebab awal terjadinya karies karena adanya variasi faktor-faktor virulensi yang khas pada bakteri yang telah diisolasi.[3] *S. mutans* merupakan bakteri anaerob yang dikenal memproduksi asam laktat sebagai bagian dari metabolismenya dan mampu melekat pada permukaan gigi dengan adanya sukrosa sebagai substrat.[3,4]

Dalam dekade terakhir ini banyak penelitian yang ditujukan untuk pengembangan tumbuhan sebagai sumber bahan obat. Untuk memenuhi keperluan perawatan kesehatan dasar, diperkirakan sekitar 75%-

80% penduduk desa di dunia menggunakan bahan obat yang berasal dari tumbuhan, dan sekitar 28% dari tumbuhan di bumi telah dipakai sebagai bahan obat tradisional.[3] Rumusan obat-obat nasional menyebutkan kurang lebih 23 negara menggunakan delima sebagai obat resmi.[4] Di Indonesia ada dua macam delima yang sering ditanam, yaitu delima merah dan putih. Delima putih sangat kaya akan kandungan alkaloid dan flavonoid. Kulit buah, kulit akar dan kulit batang delima mempunyai khasiat sebagai obat sakit gigi. Air rebusan buah dan kulit buah delima dapat dipakai sebagai obat kumur secara tradisional.

Kulit buah delima mempunyai kandungan kalium yang tinggi, selain mineral-mineral seperti fosfor, kalsium, besi, natrium, dan vitamin-vitamin seperti vitamin A, B1, B2, B3, dan C. Kalium bersama natrium, mengatur keseimbangan air di dalam tubuh dan menjaga detak jantung agar tetap normal. Kulit buah delima putih memiliki sifat antibakteri terhadap beberapa bakteri di rongga mulut.[5] Kulit buah delima mengandung senyawa alkoloid *pelletierene, granatin, betulic acid, ursolic acid, isoquercitrin, elligatanin,* resin, *triterpenoid,* kalsium oksalat, dan pati. Kulit buah delima mengandung alkaloid dan flavanoid yang mempunyai aktivitas antimikroba terhadap *S. mutans*.[3]

Penelitian ini bertujuan untuk mengetahui pengaruh ekstrak buah delima terhadap pertumbuhan bakteri *S. mutans*, dan mengetahui gambaran daya hambat ekstrak buah delima terhadap bakteri *S. mutans* dalam berbagai konsentrasi (15%, 20%, 25%, dan 30%). Hipotesis penelitian ini adalah ekstrak *Granati fructus cortex* mempunyai daya hambat terhadap *S.mutans* dan akan terdapat perbedaan efek antibakteri beberapa konsentrasi dari ekstrak *Granati fructus cortex* terhadap *S. mutans*.

METODE

Penelitian ini merupakan penelitian eksperimental laboratorium. Penelitian dilakukan di Laboratorium Penelitian Fakultas Matematika dan Ilmu Pengetahuan Alam (FMIPA) dan di Laboratorium Mikrobiologi Fakultas Kedokteran Hewan Universitas Syiah Kuala, Banda Aceh.

Bahan-bahan yang digunakan adalah: buah delima 2kg, etanol 95% 2 liter, klorheksidin glukonat 2% (Kimia Farma, Indonesia), *dimethyl sulfoxide* (DMSO), media TYS20B, NaCl 0,85%, alkohol 70%, dan biakan *S. mutans*. Alat-alat yang digunakan adalah: *rotary evaporator, disk* kosong *(Oxoid),* inkubator *(Fishers scientific),* oven (*Gallenkamp,* Jerman), kaliper geser (*Tricebramd*, China), pipet volume (*Pyrex,* Japan), ose dan kapas lidi, autoklaf (*Nacro e series model 9000-D,* Germany), cawan petri, lampu spiritus, gelas ukur (*Pyrex,* Japan), botol *vial*, pinset (*Smic*, Japan), *hot plate* (*Fisons*, UK), Pipet mikro, *pipetman starter kit* (*Gilson*, USA), rak tabung reaksi, spatula, aluminium *foil*, gelas ukur 500ml (*Pyrex*, Japan), neraca analitik (*Ohaus*), dan tabung reaksi (*Pyrex*, Japan).

Bahan-bahan pembuat media terlebih dahulu ditimbang dengan menggunakan timbangan analitik. Media TYS yang 20B digunakan: sukrosa 200g, *Yeast Extract* 10g, *Trypticase Soy Agar* 40g, *Bacto Agar* 5g, dan *Bacitrasin* 4mg/0,004g (200 UI). Setelah bahan-bahan tersebut ditimbang, dimasukkan ke dalam tabung *erlenmeyer* dan ditambahkan 1000ml aquades steril. Selanjutnya dilakukan homogenisasi dan dipanaskan di atas *hot plate* hingga mendidih. Kemudian disterilkan dalam autoklaf pada suhu 121ºC selama 15 menit. Setelah bahan agak dingin, dimasukkan ke dalam cawan petri secara asepsis menggunakan lampu spiritus.

Pembuatan ekstrak etanol buah delima

Buah delima sebanyak 2kg dibersihkan, diambil kulitnya kemudian dikeringkan selama 24 jam pada suhu kamar, dilanjutkan dengan proses maserasi dengan merendam dalam etanol menggunakan botol tertutup berwarna gelap selama 24 jam.

Hasil maserasi disaring kemudian dipekatkan menggunakan alat *rotary evaporator* pada suhu tidak lebih dari 50°C, sehingga pelarut etanol terpisah dengan ekstrak tumbuhan. Ekstrak kental yang diperoleh kemudian dimasukkan ke dalam botol steril. Konsentrasi ekstrak kulit buah delima sebanyak 15%, 20%, 25%, dan 30%, masing-masing didapat dengan melarutkan ekstrak kulit buah delima dalam larutan DMSO yang merupakan bahan alami dari serat kayu dan tidak berbahaya. Larutan DMSO berfungsi sebagai pelarut yang cepat meresap ke dalam epitel ekstrak tanpa merusak sel-sel tersebut dan sering digunakan dalam bidang kedokteran dan kesehatan. Ekstrak yang telah dilarutkan, kemudian dimasukkan ke dalam botol yang telah steril dan disimpan pada suhu 4ºC sampai waktu digunakan.

Pembiakan bakteri *S. mutans*

Bakteri yang digunakan adalah bakteri *S. mutans* dari biakan murni yang terdapat di Laboratorium Biologi FMIPA UNSYIAH. Biakan bakteri dilakukan dalam suasana aerob pada cawan petri berisi media TYS20B dan diinkubasi dalam suasana aerob pada suhu 37ºC selama 24 jam, lalu pertumbuhan *S. mutans* diamati. Apabila pertumbuhan bakteri tidak subur dan terjadi kontaminasi bakteri lain, maka prosedur pembiakan bakteri dan pengamatan diulang kembali. Sebanyak 1-2 ose dari biakan murni bakteri uji, dilarutkan dengan NaCl 0,85% sampai diperoleh kekeruhan yang sama dengan standar McFarland 0,5 ($1,5x10^8$ CFU/ml), kemudian disebarkan pada media MHA.

Uji efektifitas antibakteri metode difusi agar

Cakram kosong diambil dan diletakkan pada piring petri steril dengan menggunakan pinset steril. Empat cakram kosong digunakan untuk masing-masing bahan coba kemudian diteteskan 15µl bahan coba menggunakan pipet mikro dan didiamkan selama 60 menit. Suspensi bakteri dengan konsentrasi $1,5x10^8$ *colony forming units* (CFU)/ml diusapkan secara merata mengunakan kapas lidi pada media *Mueller Hinton Agar* (MHA) dalam piring petri. Setelah diusapkan, dibiarkan selama 30 menit supaya bakteri meresap ke dalam agar. Setelah persiapan dilakukan, cakram yang ditetesi bahan coba diletakkan pada media MHA mengikut area yang telah dibuat untuk masing-masing bahan coba. Setelah itu, media dimasukkan ke dalam inkubator pada suhu 37°C dan diamati setelah 18 dan 24 jam. Zona hambat yang terbentuk diukur dengan kaliper geser. Klasifikasi respon hambat pertumbuhan bakteri dilakukan berdasarkan metode yang telah dikembangkan sebelumnya (Tabel 1).[6]

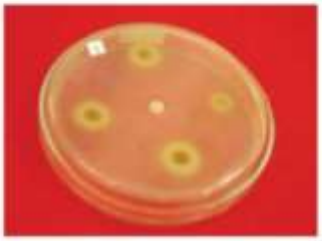
HASIL

Hasil uji daya hambat ekstrak *Granati fructus cortex* terhadap pertumbuhan *S. mutans* pada media MHA menunjukkan terbentuknya zona hambat yang berupa zona terang disekitar kertas cakram dapat dilihat pada Gambar 1. Hasil pengamatan pada konsentrasi ekstrak kulit buah delima antara konsentrasi 15% dan 25% menunjukkan adanya peningkatan rata-rata diameter zona hambat dari 10,2mm ke 13,6mm. Konsentrasi ekstrak kulit buah delima antara konsentrasi 20% dan 30% juga menunjukkan peningkatan rata-rata diameter zona hambat dari 12,8mm harus diubah ke 15,4mm. Kontrol positif tidak memiliki zona hambat rata-rata paling besar, tetapi ekstrak kulit buah delima dengan konsentrasi 30% memiliki zona hambat rata-rata yang paling besar.

Hasil analisis parametik *one-way* ANOVA memperlihatkan adanya perbedaan rata-rata diameter zona hambat yang bermakna ($p<0,05$) antara berbagai konsentrasi ekstrak kulit buah delima dalam menghambat pertumbuhan *S. mutans*. Uji Games Howe digunakan untuk melihat perbedaan yang bermakna dari variasi konsentrasi ekstrak kulit buah delima dalam menghambat pertumbuhan *S. mutans*. Uji ini menunjukan konsentrasi 15% memiliki perbedaan bermakna dengan konsentrasi 25%, 30%, dan kontrol positif. Konsentrasi 20% tidak memiliki perbedaan bermakna dengan konsentrasi 15%, 25%, 30%, dan kontrol positif. Hasil uji lanjut di atas juga menunjukkan bahwa konsentrasi 30% memang memiliki zoha hambat rata-rata yang paling tinggi (15,4mm), akan tetapi tidak memiliki perbedaan yang bermakna dengan kontrol positif yaitu 15,2mm.

Tabel 1. Klasifikasi respon hambat pertumbuhan bakteri [6]

Diameter Zona Terang	Respon Hambatan Pertumbuhan
> 20mm	Kuat
16 – 20mm	Sedang
10 – 15mm	Lemah
< 10	Tidak ada



Gambar 1. Zona hambat *S. mutans* pada kelompok kontrol dan kelompok perlakuan

DISKUSI

Penelitian ini fokus pada efek anti-bakteri berbagai konsentrasi ekstrak kulit buah delima terhadap *S. mutans*, agen kausatif utama penyebab karies pada manusia. Kemampuan bakteri ini melekat pada permukaan gigi merupakan hal terpenting bagi perkembangan karies.[1] Metode yang digunakan pada penelitian ini adalah metode tes difusi agar. Pengukuran zona hambat dilakukan setelah 18 sampai 24 jam menggunakan jangka geser. Zona hambat merupakan daerah tempat terdapatnya zona bening di sekeliling kertas cakram yang menunjukkan adanya daya hambat ekstrak kulit buah delima dan klorheksidin glukonat 0,2% sebagai kontrol positif terhadap *S. mutans*.[6]

S. mutans memiliki berbagai faktor virulensi seperti adesi, kolonisasi, dan bersifat asidofilik. Tidak seperti spesies bakteri lain yang terdapat pada plak, metabolisme bakteri menurun pada suasana pH yang rendah, namun metabolisme *S. mutans* meningkat pada pH rendah. Hal ini disebabkan karena sistem daya proton yang digunakan untuk transpor nutrisi dapat menembus dinding selnya pada pH rendah serta kadar glukosa tinggi, dan kandungan ion hidrogen yang meningkat pada keadaan asam. *S. mutans* mampu menurunkan pH rongga mulut dan mempertahankan nilai keasaman yang tidak alami. Kondisi ini menguntungkan metabolisme *S. mutans* dan tidak menguntungkan bagi spesies lain yang hidup pada waktu bersamaan.[1]

Pada penelitian ini, ekstrak kulit buah delima dibuat secara sederhana dengan menggunakan *rotary evaporator*. Ekstraksi maserasi merupakan suatu metode yang sering digunakan untuk mendapatkan senyawa dari tumbuhan

dengan menarik senyawa organik dalam suatu bahan padat menggunakan pelarut organik.9 Pelarut yang digunakan adalah etanol 95%. Proses maserasi pada kulit buah delima akan mengeluarkan senyawa-senyawa aktif yang akan diikat oleh pelarut yang digunakan. Penelitian yang telah dilakukan membuktikan bahwa kulit buah delima merupakan suatu tanaman alami yang mengandung zat-zat (misalnya alkaloid, flavonoid, dan tanin) yang dipercaya dapat menghambat pertumbuhan S. mutans dan sebagai tanaman antibakteri.[10]

Penelitian yang dilakukan sebelumnya mengungkapkan bahwa ekstrak metanol yang terdapat pada kulit buah delima mampu melawan bakteri *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi,* dan *Candida albicans*.[11] Penelitian tersebut belum menyebutkan bahwa bakteri *S. mutans* dapat dihambat pertumbuhannya oleh kulit buah delima.[11] Penelitian sebelumnya juga menunjukkan bahwa ekstrak kulit buah delima me–nempati urutan kelima dalam menghambat *S. mutans* dibandingkan dengan berbagai ekstrak herbal lainnya.[12]

Konsentrasi ekstrak kulit buah delima 15%, 20%, 25%, dan 30% kemudian dibandingkan dengan klorheksidin glukonat 0,2% sebagai kontrol positif. Klorheksidin glukonat merupakan antiseptik bersifat kimia yang banyak digunakan sebagai bahan obat kumur karena sifatnya yang dapat membunuh bakteri Gram positif serta Gram negatif. Klorheksidin bersifat netral dengan pH 5-7 di dalam rongga mulut. Interaksi ini akan mengikat permeabilitas dinding sel bakteri yang menyebabkan terjadinya penetrasi ke dalam sitoplasma. Ion kalium merupakan substansi pertama yang dapat dilihat ketika membran sitoplasma dihancurkan. Perubahan permeabilitas dari membran sitoplasma dapat menyebabkan presipitasi sari protein sitoplasma, perubahan keseimbangan osmosis, gangguan metabolisme, pemisahan sel, hambatan membran ATP, pencegahan proses anaerob, dan kematian mikroorganisme.

Hasil analisis statitik dengan *one-way* ANOVA memperlihatkan adanya perbedaan rata-rata diameter zona hambat yang bermakna ($p<0,05$) antara berbagai konsentrasi ekstrak kulit buah delima dalam menghambat pertumbuhan *S. mutans*. Hasil penelitian uji daya hambat yang dilakukan pada media padat MHA menunjukkan bahwa semakin tinggi konsentrasi ekstrak kulit buah delima maka semakin besar zona hambat yang terbentuk di sekitar kertas cakram. Beberapa faktor yang mempengaruhi adanya zona hambat bergantung kepada kemampuan difusi bahan antimikroba ke dalam media dan interaksinya dengan mikroorganisme yang diuji, jumlah mikroorganisme yang digunakan, kecepatan tumbuh mikroorganisme yang diuji, dan sensitivitas mikroorganisme terhadap bahan antimikroba yang diuji. Bahan pelarut yang digunakan juga memiliki pengaruh terhadap terbentuknya zona hambat di sekitar cakram herbal. Selain itu zat ekstraktif yang terkandung pada tumbuhan itu sendiri juga memiliki pengaruh pada daya hambat kulit buah delima terhadap pertumbuhan *S. mutans*.[13]

SIMPULAN

Dari hasil penelitian ini, dapat disimpulkan bahwa sediaan ekstrak kulit buah delima mampu secara efektif menghambat pertumbuhan *S. mutans*. Semakin tinggi konsentrasi ekstrak kulit buah delima, maka semakin besar zona hambat yang terbentuk terhadap pertumbuhan *S. mutans*. Bahan ini berpotensi sebagai obat alternatif antibakteri rongga mulut.

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Antibacterial activity of ethanolic and aqueous extracts of *Punica granatum* L. *bark*

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ABSTRACT

For thousands of years, *Punica granatum* L. has been used in traditional medicine all over the world and predate the introduction of antibacterial drugs. The aim of the present study was to investigate the antibacterial activity of aqueous and ethanolic extracts of *Punica granatum* L. bark obtained by decoction and maceration. The different extracts of *Punica granatum* L. (Lythraceae) bark have been tested for antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus, Bacillus stearothermophilus*) and Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*) by disc diffusion method. The ethanolic macerate extract showed the strong *in vitro* antibacterial activity against *Pseudomonas aeruginosa* with zone inhibition of 24.4 mm. However, the results tests by disc diffusion method revealed the effectiveness of ethanolic decoctate against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus stearothermophilus*) with diameter zone of inhibition varying with 21.1mm and 23.75 mm respectively.

Keywords: *Punica granatum* L. bark, antibacterial activity, maceration, decoction.

INTRODUCTION

The steadily increasing bacterial resistance to existing drugs is a serious problem in antibacterial therapy. *Staphylococcus aureus* is an example of bacterial resistance serial and is considered as the principal contaminant of clinical infections. Recently, the acceptance of traditional medicine as an alternative form for health care and the development of bacterial resistance to the available antibiotics has led authors to investigate the antibacterial activity of medicinal plants (Scrinivasan , 2001; Kumarasamy, 2002; Ali, 2001; Masika and Afolayan, 2002; Hamill, 2003; Shah, 2005; Lahlou, 2009). Plants and plant derived agents have long history to clinical relevance as source of potential chemotherapeutic agents (Cushnie *et al.*; 2005). Thousands of plant species have been screened for their antimicrobial activity, but relatively few were found to be sufficiently active (Poyart -Salmeron, 1990; Meng, 2000) and non toxic to humans (Izzo, 2004). The tree of *Punica granatum* L. (Lythraceae) is extensively abundant in South-West of Algerian Sahara. The different parts of this plant such as flowers, seeds and bark have been employed against inflammatory and infectious pathologies. The purpose of the present study was to investigate the antibacterial activity of bark extracts of *Punica granatum* against Gram-positive and Gram-negative bacteria. The selected bacteria were antibiotic resistant or multi-resistant human pathogens. The extracts with the highest antibacterial effectiveness were chosen for subsequent use in pharmaceutical formulations.

MATERIALS AND METHODS

Plant material

The plant used for the present study was collected in September 2010 in Timimoun area, Adrar Department, Algeria. The bark was separated from fruits and dried at room temperature for 12 days. The dried bark was milled to a fine powder in an electrical mill and stored in the dark at room temperature in closed containers until required.

Preparation of *Punica granatum* extracts

Maceration: About 5 g of powdered material was macerated with 20 ml of ethanol for 30min. The ethanol extract was filtered using whattman filter paper and then concentrated under vacuum at 65°C using a Rotary Evaporator. The residue obtained was stored at °4 C prior to analysis.

Decoction: Dried and powdered plant material 5 g was boiled with 50 ml of water for 30 min. After cooling, the mixture was filtered and stored to 4°C prior to analysis.

Similar protocols were used for preparation of ethanol decoctate and aqueous macerate.

Microorganisms and media

The antibacterial activity of ethanol and aqueous extracts of *Punica granatum* bark were evaluated using the following strains of bacteria, Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25922), *Bacillus stearothermophilus* (ATCC 11778), Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853). These bacterial strains were obtained from the Pasteur Institute, Algiers, Algeria.

The strains were identificated by the use of Biochemical profiles according to the recommendations of the Manual of Clinical Microbiology (Murray *et al.*; 1999). All organisms were maintained in brain-heart infusion (BHI medium) containing 30% (v/v) glycerol at -20°C. Before testing, the suspensions were transferred to trypticase soy agar supplemented with 5% of sheep blood (Difco) and aerobically grown overnight at 35°C. Individual colonies were isolated and suspended in 5 ml of 0.9% NaCl solution. The inocula were prepared by adjusting the turbidity of the suspension to match the 0.5 McFarland standard and diluted in CAMHB (cation - adjusted Müeller–Hinton broth) in order to achieve the adequate inoculum in each case.

The cell number in CAMHB was estimated using a serial dilution technique (NCCLS, 2002) for each assay.

Disk diffusion method

Petri dishes (90 cm in diameter) were prepared with 10 ml of a base layer of Müeller–Hinton gelose medium (MHG) and inoculated with 100 µl of each bacterial suspension (velickovic *et al*; 2003). After drying in a sterile hood, 6 mm diameter disks soaked with aqueous decoctate 5.5 µl; ethanolic decoctate 5.8 µl; aqueous macerate 5.4 µl and ethanolic macerate 5.7µl. The different extract dilutions were placed on the gelose. The dishes were incubated at 35°C for 24hours. All tests were performed in duplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced.

RESULTS AND DISCUSSION

The results of the disk diffusion test indicated that crude ethanol and aqueous extracts of *Punica granatum* bark showed different degrees of growth inhibition, depending on the bacterial strains (Tables 1 and 2). The aqueous and ethanol decoctate extracts showed the broadest antibacterial activity by inhibiting growth of all bacterial strains tested (the diameter of inhibition zone, 15.85-22.85 mm and 21.30-23.75 mm respectively). Of all ethanol and aqueous macerate extracts tested, the aqueous macerate extract showed the highest antibacterial activity against some strains, such as *Pseudomonas aeruginosa* (24.4mm), and *Escherichia coli* (23.3mm; 23.85 mm) respectively for aqueous and ethanol macerates.

Table 1: Antibacterial activity of the ethanolic and aqueous decoctates of *Punica granatum* bark by disc diffusion method.

Microorganisms	Aqueous decoctate	Ethanolic decoctate
	Inhibition zone (mm)	Inhibition zone (mm)
Gram-positive bacteria		
Staphylococcus aureus	19.00	21.30
Bacillus stearothermophilus	15.85	23.75
Gram-negative bacteria		
Escherichia coli	20.40	22.10
Pseudomonas aeruginosa	22.85	22.75

Table 2: Antibacterial activity of the ethanolic and aqueous macerates of *Punica granatum* bark by disc diffusion method.

Microorganisms	Aqueous macerate	Ethanolic macerate
	Inhibition zone (mm)	Inhibition zone (mm)
Gram-positive bacteria		
Staphylococcus aureus	23.05	20.90
Bacillus stearothermophilus	21.20	21.05
Gram-negative bacteria		
Escherichia coli	23.30	23.85
Pseudomonas aeruginosa	24.40	19.45

The disc diffusion bioassay showed that bark decoctate extracts have the highest activity against all Gram-positive bacteria and they also showed good activity against Gram-negative bacteria. The reason for different sensitivity between Gram-positive and Gram-negative bacteria could be ascribed to the morphological differences between these microorganisms. Gram-negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da (Nikaido and Vaara, 1985). The Gram-positive bacteria should be more susceptible since they have only an outer peptidoglycan layer which is not an effective permeability barrier (Scherrer and Gerhardt, 1971).

Based on inhibition zone values, bark aqueous macerate showed to be potent inhibitors against all bacterial strains.

Same results were obtained via the bark ethanolic macerate with different zone diameter of inhibition. These results are in agreement with those reported by Curtay and Jung (2010). They found that *Punica granatum* bark have to be *in vitro* antibacterial activity.

These results suggest that the inhibitory effect exhibited by the macerate and decoctate crude extracts of *Punica granatum* bark may be attributable to the tannins that represent 28% of bark constituents (Blansky and Newman, 2007). This family of compounds has been found *in vitro* to have various pharmacological properties such as antioxidant, antimicrobial and anti-inflammatory (Curtay and Jung, 2010). Furthermore, the observed antibacterial activity may be due also to other secondary metabolites like phenolic compounds and saponins. In general, we observed that the ethanol decoctate extract was more efficacy than aqueous extract because ethanol allowed to extract well the less polar compounds such as terpenic derives (Emmanuel *et al*; 2002).

The obtained results might be considered sufficient to further studies for the isolation and identification of the active principles and to the evaluation of possible synergism among extract components for their antimicrobial activity. Investigations are in progress to determine the degree of toxicity of these extracts.

CONCLUSION

Extracts of *Punica granatum* L. bark in this study demonstrated a broad-spectrum of activity against both gram-positive and gram-negative bacteria with different diameter zone of inhibition. The broad-spectrum antibacterial activities of the plant extract, possibly due to the secondary metabolites such as tannins, phenolic compounds or saponins that were abundant in this plant. This study paves the way for further attention and research to identify the active compounds responsible for the plant biological activity. Further studies should be undertaken to elucidate the exact mechanism of action by which extracts exert their antibacterial effect.

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Test the effectiveness of antibacterial effect of the skin of the pomegranate fruit (*Punica granatum*) extract against the growth of *Escherichia coli in vitro*

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ABSTRACT

Background: Pomegranate (*Punica granatum*) is one of the traditional medicines that contains antibacterial compounds that are effective against bacterial growth. Its chemical content includes saponins, tannins and flavonoids. Escherichia coli is a gram-negative bacterium which is a normal flora germ found in the human large intestine. These bacteria are pathogenic when they are outside the intestine and produce enterotoxins in epithelial cells which cause diarrhea. The purpose of this study was to determine the antibacterial effect of pomegranate skin extract on the growth of *Escherichia coli*.
Methods: This study used an experimental design study with a complete randomized design study divided into 5 groups namely groups 1 (15 ug/ml), 2 (20 ug/ml), 3 (25 ug/ml), positive control (ciprofloxatin), negative control. Making pomegranate peel extract was done by maceration method then rotary, after that the effectiveness of pomegranate extract extracted by the diffusion method was tested using Anova one-way test.
Results: The results showed that the extract of pomegranate peel showed that it was able to inhibit the growth of *Escherichia coli* with a ratio of constants (15 ug/ml, 20 ug/ml, 25 ug/ml with inhibition diameters of 6.7 mm, 6.7 mm, 6, 7 mm, while for positive control with ciprofloxatin showed bacterial resistance to antibiotics.
Conclusions: Statistically, pomegranate skin extract has an antibacterial power which is meaningful with p 0.005. Pomegranate rind extract has antibacterial activity against *Escherichia coli* medium because inhibition zone is 5-10 mm.

Keywords: Antibacterial, *Escherichia coli*, Pomegranate

INTRODUCTION

Diarrhea is a major cause of morbidity and mortality in children under five, estimated at about 1.3 million deaths of children under five years of age occur all over the world. The prevalence of high incidence of diarrhea in Indonesia in the year 2018 occurs in young toddlers (age 1-5 years), namely by 6.7%.[1] Morbidity and mortality of diarrhea in Indonesia is still high.

Based on a survey of morbidity carried out by the Ministry of Health of the year 2010 s/d 2018 noticeable trend of increased incidence of diarrhea. Percentage of the morbidity of diarrhea in the year 2010 is 37.4%, then increased to 42.3% in 2012, and decreased by 41.1% in year 2013.[2] The percentage of cases of diarrhea in children under five in the Province of West Sumatra in the year 2018 by 31.400 cases (department of health of West Sumatra Province, 2018). Diarrheal diseases still occupy the order of 10 common diseases in the City Padang.[3]

Escherichia coli is often the cause of infection of the urinary tract, biliary tract and other places in the

abdominal cavity. *Escherichia coli* is the cause of diarrhea and tract infections kemih.[4] *E. coli* belongs to the bacteria heterotrophs that obtain food in the form of substances organic from the environment because cannot draw up its own organic substances that it needs. These bacteria are pathogenic if they are outside the intestines and produce enterotoxins which cause diarrhea and are associated with enteropathogenic produce enterotoxins on the cell epitel.[5]

Infectious diarrhea caused by bacteria can actually be done with natural ingredients, namely, insufficient fluid intake of the body. However, if diarrhea cases are settled more than two days it is recommended to do a medical consultation. Usually the doctor will give you antibiotics if the diarrhea is caused by bacteria.[6] Administration of antibiotics that are not rational can trigger the occurrence of bacterial resistance. Bacteria that are resistant to antibiotics also caused due to the use of antibiotics which is increasingly extends.[7] Therefore, with the presence of bacteria that are resistant to antibacterial will encourage the importance of utilization of herbal remedies in natural.[8]

Plants that can be used as a herbal medicine d acts as an antibacterial is the pomegranate (*Punica granatum* L.).[9] The content of antibacterial namely alkaloids, saponins, and flavonoids are potential as kemoprotektif and is able to inhibit the lipid peroxide is non-enzymatic. The higher the levels of flavonoids, the potential antioxidant will be higher. Mechanism of action of flavonoids as antibacterial compounds are divided into three, namely inhibiting the synthesis of nucleic acids, inhibits the function of cell membranes, and inhibit the metabolism energy.[10] Alkaloids are nitrogen compounds heterocyclic containing at least one nitrogen atom and is alkaline. The cluster bases will react with the acidic compounds that exist in bacterial cells such as DNA, which is the main constituent of the cell nucleus. With the disruption of the DNA, then the synthesis of proteins and nucleic acids in the cells will disturbed.[11]

Because of the usefulness of this, pomegranate has a lot of be used as one of the alternative treatment traditional.[12] Each active substance has a different mechanism as an antibacterial. Previous research has shown that the methanol extract of pomegranate peel has been shown to have activity against the bacteria *Shigella dysenteriae, Staphylococcus aureus, Pseudomonas aeruginosa* and *E. coli*.[13] The purpose of this study was to determine the antibacterial effect of pomegranate skin extract on the growth of *E. coli*.

METHODS

This study used an experimental design study with a complete randomized design study divided into 5 groups, group 1 (25 ug/ml), 2 (20 ug/ml), 3 (15 ug/ml), positive control (Ciprofloxatin 500 mg), negative control (aquades). Ciprofloxatin 500 mg dosage form was suspension powder that was liquefied into 1 cc of sterile aquades. After that the antibiotic disk was dissolved. Making pomegranate peel extract was done by maceration method then rotary, after that the testing of the effectiveness of pomegranate peel extract was done by diffusion method by using ANOVA one-way test.

This research was conducted at the regional health laboratory of West Sumatra Province. Held on 4th November - 7th December 2019. With inclusion criteria ripe pomegranate skin. Making pomegranate rind extract was done by maceration method then rotary, after that the testing of the effectiveness of pomegranate extract was carried out by diffusion method.

RESULTS

The results of the research can be seen in Table 1 based on the calculation that has been done. Based on the calculation that has been done, the average value of the diameter of the inhibition zones in group 1 at 6.7 mm, group 2 of 6.7 mm, group 3 by 7.7 mm, and the negative control group and positive control do not have the power resistor because the average value of the diameter of the inhibitory zone of 0.00 mm.

Table 1: Inhibition zone diameter of pomegranate rind extract (*Punica granatum*) against *Escherichia coli* bacteria by diffusion method.

Treatment	Deuteronomy			Average (D*)
	1	2	3	
Positive control	0	0	0	0
Negative control	0	0	0	0
1 (15 ug/ml)	6 mm	7 mm	7 mm	6.7 mm
2 (20 ug/ml)	6 mm	7 mm	7 mm	6.7 mm
3 (25 ug/ml)	7mm	8 mm	8 mm	7.7 mm

DISCUSSION

The results of the study showed that the average value of the diameter of the inhibition zone of one group of 6.7 mm, a group of two 6.7 mm, a group of three 7.7 mm, negative control group and positive not have a zone of inhibition. In the positive control group was given treatment with the antibiotic ciprofloxacin, the zone of

inhibition is not formed. It is shown that ciprofloxacin is not likely to provide the effect of the inhibition at low concentrations. So that confirms that the results of the antibiotic ciprofloxacin resistance to bacteria *E. coli*. Antibiotics group florokuinolon the most widely used for the treatment of infections is ciprofloxacin, especially that caused by gram-negative bacteria, especially *E. coli*.[14] Resistance *E. coli* to the antibiotic ciprofloxacin is generally caused by chromosomal mutations in the genes gyr A and par C. However, recent research shows that resistance to a low level can also be mediated by plasmids through the acquisition hen qnr-mediated by plasmids pMG252.[15] Increased resistance of *E. coli* to the antibiotic ciprofloxacin has been widely reported. Other studies reported as much as a 20,65% isolates of *E. coli* resistant against ciprofloxatin among the 155 isolates clinic *E. coli* in Pakistan. A specimen of urine patients with UTI in RSUD Abdoel Moeloek (RSUDAM) Lampung Province and obtained 30 isolates positive for *E. coli*.[16] Research in Makassar found 48% isolates of *E. coli* resistant ciprofloxatin among the 39 isolates positive for *E. coli*.[17]

According to the WHO (2012), inaccuracy as well as not rational the use of antibiotics is the cause of most of the main spread of resistant microorganisms. So, a drug effective for the treatment, then it should reach the place of its activity in the body with accuracy and a sufficient amount to produce a concentration of effective.[18] Antibiotics will experience transportation is dependent with the power process to plasma proteins. The form that is not bound by such a protein which is pharmacologically active, have the ability as antibakteri.[19] Other mechanisms that cause resistance is decreased accumulation of the drug in the cells by the increase in the pump efflux to native and decreased membrane outer porins.[20] Some species of *Enterobacteriaceae*, including bacteria *E. coli* have a chromosomal native pump efflux AcrAB-To1C which belongs to the families RND (resistance-nodulasi division).[21]

The criterion of strength antibacterial power is divided into negative control group did not have a zone of inhibition from the results, the look the higher the concentration the inhibition pomegranate the larger the diameter of the inhibition zone formed. The inhibition zone diameter of 5 mm or less is categorized weak, the diameter of the inhibition zone 5-10 mm categorized as moderate, the diameter of zone of inhibition of 10–20 mm are categorized powerful and the inhibition zone 20 mm or more are considered very strong.[22] This means that the bacteria *E. coli* sensitive to extracts of pomegranate peel because the results of inhibition the greater the in accordance with the high concentration given.

The inhibition zone formed around the disk that has spilled fruit extract pomegranate red shows that the extract contains active compounds that are as antibacterial.[23] The content of the antibacterial contained in pomegranates, namely alkaloids, saponins, and flavoid potential as kemoprotektif and is able to inhibit the lipid peroxide is non-enzymatic. The higher the levels of flavonoids, the potential antioxidant will be higher.[24] Because of the usefulness of this, pomegranate has a lot of to uses one of the alternative treatments traditional each active substance has a different mechanism as an antibacterial. Mechanism of action of flavonoids as antibacterial compounds are divided into three, namely inhibiting the synthesis of nucleic acids, inhibits the function of cell membranes, and inhibit the metabolism energy.[25]

The mechanism in inhibiting the synthesis of nucleic acids is to inhibit the formation of DNA and RNA through the ring A and B which play a role in hydrogen bonding. This leads to the buildup of bases of nucleic acid, and the occurrence of damage to the permeability of the bacterial cell wall, lysosomes, as well as mikrosom.[26] The mechanism in inhibiting the function of cell membranes is by forming complex compounds with proteins of the extracellular and dissolved which cause damage to bacterial cell membranes and followed by a discharge of compound is intracellular. While the mechanism of flavonoids in inhibiting energy metabolism is to inhibit the cytochrome C reductase and inhibits the use of oxygen on the bacteria. Whereas the energy needed bacteria in the conduct of the biosynthesis of macromolecules.[27] Alkaloids are nitrogen compounds heterocyclic containing at least one nitrogen atom and alkaline Cluster bases will react with the acidic compounds that exist in bacterial cells such as DNA, which is the main constituent of the cell nucleus. With the disruption of the DNA, then the synthesis of proteins and nucleic acids in the cells will disturbed.[28]

CONCLUSION

Statistically the ethanol extract of pomegranate peel has antibacterial power that meaningful with p 0.005. The ethanol extract of pomegranate peel has antibacterial activity against the bacteria *Escherichia coli* was due to the inhibitory zone of 5-10 mm.

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